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Repeated hexanucleotide C-C-C-C-A-A is present near free ends of macronuclear DNA of *Tetrahymena*

(ciliate/differentiation/genome alteration/exonuclease/chromosome breakage)

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ABSTRACT **The tandemly repeated hexanucleotide C-C-C-C-A-A has previously been found near the termini of extrachromosomal gene coding for ribosomal RNA as well as in many other locations of the genome of *Tetrahymena*. Moreover, the organization of these clusters of repeats in the somatic macronucleus is different from that in the germinal micronucleus. In this study we used the exonuclease Bal 31 to show that the repeats are located near free ends of DNA in the macronucleus. When whole cell DNA or macronuclear DNA was digested with Bal 31 to remove approximately 600 base pairs from free ends, 80% of the C_4A_2 repeats were removed, as judged by hybridization. Because no particular cluster was resistant to exonuclease digestion, we believe that essentially all the C_4A_2 repeats are located near free ends of DNA. The C_4A_2 repeats in the micronucleus, on the other hand, were not digested by Bal 31.**

The ciliated protozoan *Tetrahymena thermophila* normally contains a macronucleus and a micronucleus in each cell. During conjugation the macronucleus degenerates and the micronucleus goes through a series of events to produce the new macronucleus and micronucleus for the following asexual generation. Thus, the two nuclei share the same genetic origin, but it is the micronucleus that maintains the genetic continuity of the organism (1). Recent studies have shown that the genome of this organism is significantly altered during the formation of the macronucleus (2). The gene coding for ribosomal RNA (rDNA) is selectively amplified several hundredfold (3), and about 15% of the genome is eliminated (4, 5).

Another observation related to genome alteration has been made recently. In the macronucleus the amplified rDNA exists as extrachromosomal palindromic molecules (6, 7). A tandemly repeated hexanucleotide, $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_{20-70}$, was found at or near the free ends of this linear molecule (8). This repeated sequence was later found in other locations of the genome (3, 9). It exists in many clusters in both the macronucleus and micronucleus. After restriction enzyme digestion the DNA fragments containing this repeated sequence are found to be of different sizes in these two nuclei. These results suggest that the genome is somehow altered in regions associated with the C_4A_2 repeats.

In this report we present evidence which reveals the nature of this alteration. Using the exonuclease Bal 31 we were able to show that the C_4A_2 repeats are located near free DNA ends in the macronucleus. These free ends are apparently generated through chromosome fragmentation during development. Thus in *Tetrahymena*, as in two other ciliates (10, 11), the simple repeated sequences seem to play a significant role in the fragmentation of chromosomes.

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MATERIALS AND METHODS

Cells and Culture Conditions. *T. thermophila* inbreeding strain B, obtained from P. Bruns (Cornell University), was used throughout this study. The cells were cultured in axenic media as described (12). Macronuclei and micronuclei were isolated from cells in late logarithmic phase of growth by using the method of Gorovsky *et al.* (12).

DNA Isolation. DNAs were isolated from macronuclei and micronuclei as described (13). For isolation of high molecular weight DNA, the nuclear lysate or the whole cell lysate was sedimented through a discontinuous sucrose gradient which contained equal volumes of 15%, 25%, and 30% sucrose in 0.5 M NaCl/10 mM Tris·HCl, pH 7.4/1 mM EDTA. The gradient was centrifuged in a Beckman SW 27 rotor at 20,000 rpm for 16 hr. Fractions were collected and the DNA in each fraction was determined by electrophoresis in a 0.5% agarose gel. The rDNA was usually found between the 15% and the 25% sucrose layers. The bulk of the DNA was sedimented to the bottom of the gradient and was purified by phenol extraction.

Enzyme Digestion and Gel Electrophoresis. The *Eco*RI and the exonuclease Bal 31 were purchased from New England BioLabs. The digestion conditions recommended by the supplier were used. Digestion with Bal 31 was as described (13). About 3 μg of DNA was digested with 1 unit of the enzyme, and the reaction was terminated by the addition of EDTA and chilling in ice. The DNA was extracted with phenol and precipitated with ethanol before *Eco*RI digestion. At least a 2-fold excess of *Eco*RI was used to ensure complete digestion. Gel electrophoresis was carried out in 1.0% agarose in a horizontal slab gel apparatus as described (13).

DNA Labeling and Hybridization. Clone pTt 220b (13) was labeled by nick-translation (14). The C_4A_2 repeat of rDNA was labeled by a modified method of nick-translation as described (8). α-^{32}P-Labeled dATP and dCTP (Amersham; 400 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were used as precursors. About 5×10^5 cpm of labeled DNA per ml was used in the hybridization. Hybridization with C_4A_2 repeats was carried out at 65°C for 10–18 hr in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0/0.1 M Tris·HCl, pH 7.4/0.1% NaDodSO$_4$/Denhardt solution. Hybridization with pTt 220b was done under the same condition except that 0.6 M sodium chloride and 0.06 M sodium citrate were used. The hybridized filter was washed extensively with 0.3 M sodium chloride/0.03 M sodium citrate at 65°C and detected by autoradiography on Kodak XR-5 x-ray film with a Du Pont intensifying screen. For quantitation the hybridized filter was sliced and assayed in toluene-based scintillation fluid in a liquid scintillation counter.

Abbreviations: rDNA, DNA coding for ribosomal RNA; kb, kilobase(s).

RESULTS

It has been shown recently that chromosome breakage occurs in *Tetrahymena* to separate the rDNA from its right flanking sequence during macronuclear differentiation (13). The free DNA ends thus generated are sensitive to the exonuclease Bal 31 (13). This exonuclease is known to digest both double- and single-stranded DNA from both the 5′ and the 3′ ends (15). If chromosome breakage indeed occurs at or near all the C_4A_2 repeats in the genome (5), these repeats should become adjacent to free DNA ends in the macronucleus and also may be sensitive to Bal 31. To test this idea the following experiments were performed. High molecular weight DNA was isolated from whole cell lysate, which contained mainly macronuclear DNA. The majority of the extrachromosomal rDNA was removed by sedimentation in a sucrose gradient. The remaining DNA was treated with Bal 31 for various lengths of time to digest the exposed ends. It was then cleaved with *Eco*RI, separated by electrophoresis in an agarose gel, and blotted for hybridization. These experiments are diagrammed in Fig. 1.

To determine the extent of Bal 31 digestion, one set of blotted DNA was hybridized with the clone pTt 220b. This clone contains a portion of the right flanking sequence of rDNA from the micronucleus. The same sequence is adjacent to a free DNA end in the macronucleus (13). As shown in Fig. 2, the flanking sequence is in a 3.0-kilobase (kb) *Eco*RI fragment in the macronucleus (13). The exonuclease progressively shortened this fragment from the free end, as expected. The number of nucleotides removed at each time point can be roughly estimated from the changes in the fragment size. It was found that about 0.04, 0.30, 0.58, 0.88, and 1.29 kb of DNA was removed after 1, 2, 3, 5, and 10 min of digestion. These numbers were taken as indications of how much DNA may also be removed from other free DNA ends under the same conditions.

To find out whether the C_4A_2 repeats are also sensitive to Bal 31 treatment, another set of blotted DNA was hybridized with

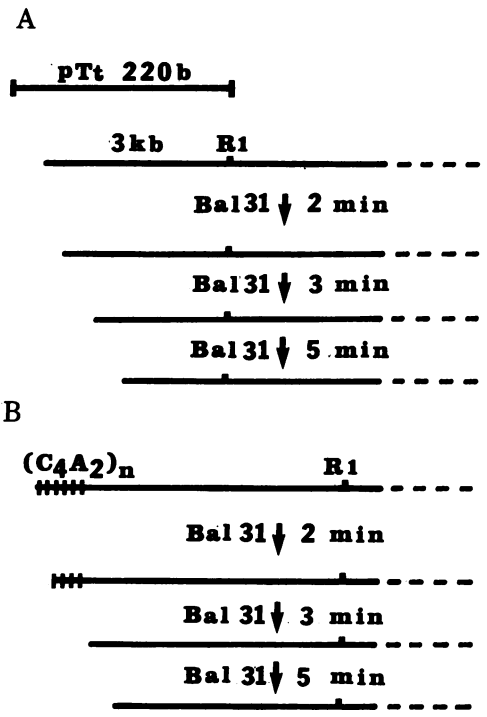

FIG. 1. Bal 31 digestion of free DNA ends. In *Tetrahymena* the DNA flanking the right side of rDNA in the micronucleus is separated from the rDNA in the macronucleus by a chromosome break. This break creates a free DNA end next to the flanking sequence. (*A*) Flanking sequence is represented by a solid line, with the free end on the left. Digestion with Bal 31 shortens this molecule progressively from the free end. This change can be detected by the shortening of the 3-kilobase (kb) *Eco*RI fragment that contains this sequence. The clone pTt 220b contains 3.5 kb from this region of the micronuclear DNA and can be used as a probe to detect the flanking sequence by Southern hybridization. If C_4A_2 repeats in the macronucleus are also located near free ends of DNA, they also may be sensitive to Bal 31. (*B*) One such cluster of C_4A_2 repeat is shown, represented by vertical lines at the left end of the DNA. After brief treatment with Bal 31 (2 min) part of the C_4A_2 repeat may be removed. This fragment may still hybridize with the C_4A_2 repeat although it should be slightly smaller. Longer digestion (5 or 10 min) would probably remove the repeat completely and the fragment should no longer hybridize with the C_4A_2 repeat.

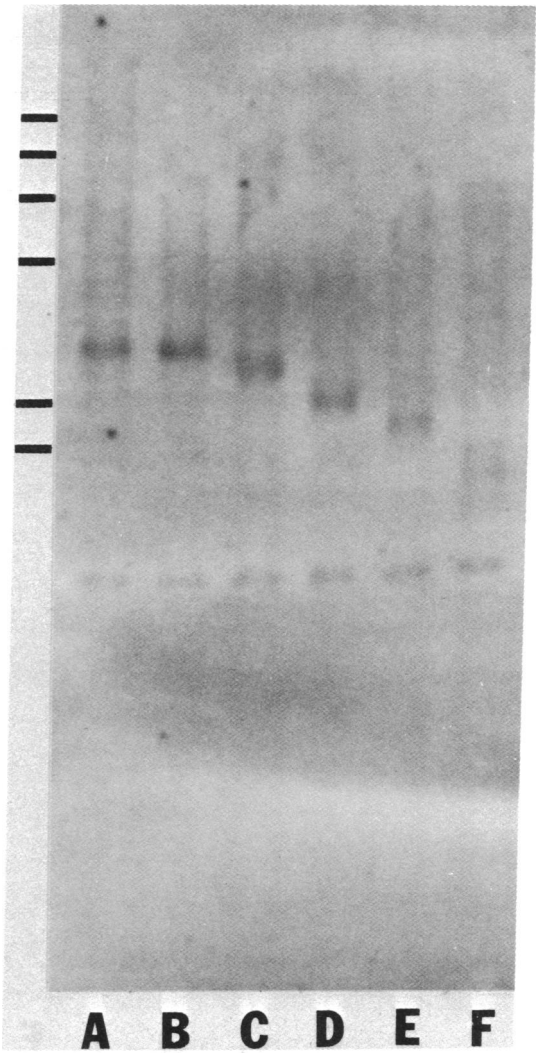

FIG. 2. Bal 31 digestion of the free end of rDNA flanking sequence. High molecular weight whole cell DNA which contains mainly macronuclear DNA was digested with Bal 31 for varying lengths of time. The DNA was then digested with *Eco*RI, separated in a 1.0% agarose gel, and blotted onto a filter for hybridization. To estimate the extent of digestion, the clone pTt 220b was used to hybridize the filter. The clone contained a part of the right flanking sequence of rDNA, which was adjacent to a free end in the macronucleus. Without Bal 31 treatment, a 3.0-kb fragment was detected. Bal 31 shortened this fragment progressively from the free end. The reductions in size were estimated to be 0.04, 0.30, 0.58, 0.88, and 1.29 kb after 1, 2, 3, 5, and 10 min of digestion. The minor band in the lower portion of the gel could be a homologous sequence from some other portion of the genome. Lanes: A, DNA not treated with Bal 31; B, C, D, E, and F, DNA digested with Bal 31 for 1, 2, 3, 5, and 10 min, respectively. Bars at left show positions of the six large *Hin*dIII fragments of phage λ DNA in the same gel as size markers: 23, 9.1, 6.3, 4.2, 2.2, and 1.9 kb.

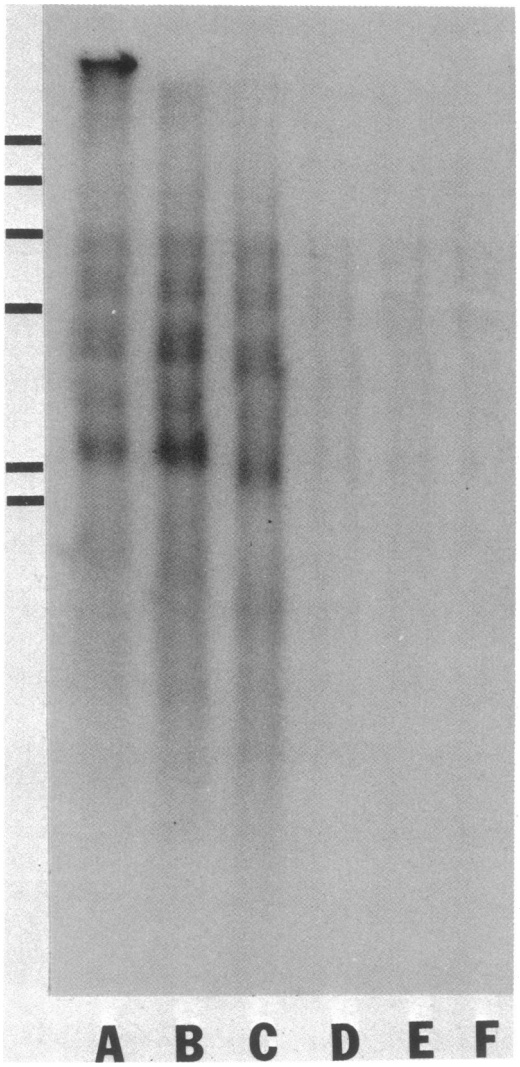

FIG. 3. Digestion of macronuclear C_4A_2 repeats with Bal 31. To determine the sensitivity of macronuclear C_4A_2 repeats to Bal 31, the filter containing the digested DNA described in Fig. 2 was hybridized with C_4A_2 repeats prepared from rDNA. The effect of the exonuclease could be seen after 2 min of digestion, when most of the bands had decreased slightly in size (lane C). Digestion for 3 min or longer removed essentially all C_4A_2 repeats from the DNA (lanes D, E, and F). The black bars represent *Hin*dIII fragments of phage λ DNA as size markers. The extent of hybridization in each individual band was measured directly by assaying the filter strip in a liquid scintillation counter. Lanes A–F were found to contain 13,300, 15,300, 12,300, 3500, 3200, and 2900 cpm. The background hybridization in the lane containing λ DNA was 450 cpm.

the C_4A_2 repeats of the extrachromosomal rDNA (Fig. 3). It is clear that the C_4A_2 repeats in the macronucleus can be removed by Bal 31 digestion. After 2 min of treatment the hybridized bands began to decrease in size (compare lanes B and C). Longer digestion eliminated the hybridization almost completely.

To estimate the amount of hybridization with the C_4A_2 repeats, the hybridized filter shown in Fig. 3 was cut into strips containing individual lanes and assayed in a liquid scintillation counter. From these measurements it was estimated that about 76% of the hybridization was lost after 3 min of Bal 31 digestion, and about 81% was lost after 10 min of digestion. Similar results were also obtained from a separate experiment. In this experiment the macronuclear DNA was used, and the digestion was carried a little further. It was found that about 82%, 90%, and 92% of the hybridization was removed after 2.5, 5, and 10 min of Bal 31 digestion. Under the same conditions the rDNA flanking sequence was shortened by 1.1, 1.4, and 1.9 kb. From these estimates it is clear that about 80% of the C_4A_2 repeats are lost when about 600 base pairs of DNA is removed from the end. An additional 10% can be removed after further digestion.

The micronucleus of *Tetrahymena* also contains many clusters of C_4A_2 repeats (3, 9). Presumably most of these repeats are located in the internal regions of the chromosome and thus should not be sensitive to Bal 31 digestion. To verify this, high molecular weight DNA was isolated from the micronucleus, digested with Bal 31, and analyzed by Southern hybridization. After extensive digestion with Bal 31, little change was observed in the C_4A_2 repeats of the micronucleus (Fig. 4). The few bands

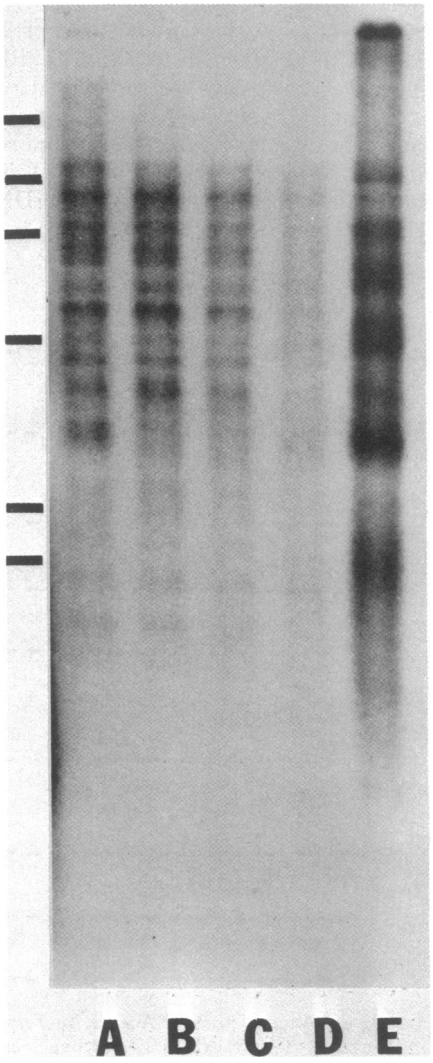

FIG. 4. Micronuclear C_4A_2 repeats are not digested by Bal 31. To determine the sensitivity of micronuclear C_4A_2 repeats to Bal 31, high molecular weight micronuclear DNA was digested with Bal 31 for 10 min and hybridized with C_4A_2 repeats after *Eco*RI digestion, gel electrophoresis, and blotting as in Fig. 2. Lanes: A, micronuclear DNA not treated with Bal 31; B, the treated sample (the only band that was clearly sensitive to Bal 31 was probably derived from contaminating extrachromosomal rDNA); C, equal amounts of whole cell and micronuclear DNA which were mixed and digested with Bal 31 under the same conditions (banding pattern was essentially the same as that of digested micronuclear DNA); D, high molecular weight whole cell DNA digested with Bal 31 under the same conditions; E, untreated sample. It is clear that under this condition most of the C_4A_2 repeats were removed from the macronucleus. The black bars represent *Hin*dIII fragments of phage λ DNA as size markers.

that disappeared probably were derived from macronuclear contamination which included the extrachromosomal rDNA. Under the same digestion conditions, most of the C_4A_2 repeats were removed from the whole cell DNA.

The contrast between these two nuclei was better illustrated in a mixing experiment. In this experiment, equal amounts of the whole cell and the micronuclear DNA were mixed together and treated with Bal 31. The treated DNA was then digested with *Eco*RI and analyzed by Southern hybridization as before. The hybridization pattern of this DNA was indistinguishable from that of the micronuclear DNA after Bal 31 digestion. This result indicates that only the C_4A_2 repeats of the macronucleus are sensitive to the digestion by Bal 31. Thus, it is clear that the C_4A_2 repeats in the micronucleus are not in locations susceptible to exonuclease digestion. The sensitivity of these sequences to Bal 31 in the macronucleus therefore must be derived during differentiation.

DISCUSSION

Using the exonuclease Bal 31 we have shown that the majority of the C_4A_2 repeats in the macronucleus of *Tetrahymena* are located near free DNA ends. The quantitative estimates of this study suggest that roughly 90% of these repeats are located less than 2 kb from free ends, and about 80% of them are probably within the first 600 base pairs from free ends. After prolonged digestion all the bands seem to decrease equally in hybridization intensity. The residual hybridization is essentially the same in banding pattern as the nontreated sample (data not shown). From this result it is also clear that most of these repeats are equally sensitive to Bal 31. Thus, it is likely that all the DNA clusters of C_4A_2 repeats in the macronucleus are located near free DNA ends, although only 90% of the molecules containing each cluster are digested by Bal 31.

Besides the exonuclease activities, Bal 31 is also known to have an endonuclease activity for single-stranded DNA (15, 16). For this reason it could be argued that the C_4A_2 repeats were sensitive to Bal 31 due to the presence of single-stranded regions in the repeats. However, we do not think that this is the case. If single-stranded regions were indeed responsible for the sensitivity, the DNA would have to be broken at these regions before the repeats were removed by the exonuclease activity. The breakage would reduce the fragment size drastically and thus change the overall banding pattern significantly. We did not observe any drastic change in banding pattern. Rather, we found that the fragments were shortened slightly before they disappeared (Fig. 3, lane C). This is exactly what one would have expected if the repeats are indeed located near free ends.

Because the C_4A_2 repeats were not digested by Bal 31 in the micronucleus, the repeats must be present in locations not susceptible to exonucleases, such as the internal regions of the chromosomes. Thus, the sensitivity of these sequences in the macronucleus must be acquired during development and is not an inherited property of the repeats. At least two processes can be imagined to account for this change. Site-specific breakages could occur at or near these repeats in the chromosome to produce DNA fragments with C_4A_2 repeats at the ends (5). Alternatively, the repeats could be translocated to the free ends of DNA, which could be the existing telomeres, if they are sensitive to Bal 31 digestion, or to new ends generated through site-specific breakage. It is unlikely that all the C_4A_2 repeats are moved to the telomeres. There are only 10 telomeres in *Tetrahymena*, and there are roughly 200 clusters of C_4A_2 repeats in the micronucleus (9). To put all the repeats in these 10 locations would make each cluster long. Even if the telomeric DNA were sensitive to Bal 31 digestion, this arrangement would not likely give the banding pattern of the C_4A_2 repeats we have found in the macronucleus or the Bal 31 digestion kinetics observed. Chromosome breakage, on the other hand, has been shown to occur near rDNA during development (13). This study suggests that the breakage also occurs in many other locations in the genome to generate the free ends of DNA at which the C_4A_2 repeats are located.

Although chromosome breakage probably is involved to render the C_4A_2 repeats susceptible to exonuclease digestion, it is not clear whether the repeats actually serve as the breakage sites for chromosome fragmentation. It is still possible that the C_4A_2 repeats are translocated to the free ends after breakage occurs.

If chromosome breakage indeed occurs, the macronuclear DNA should be smaller than an intact chromosome. The size of the macronuclear DNA has been measured in several studies (17–19). In one case it was shown to be near the size of the chromosome (4×10^4 kb) (17). In two others it was considerably smaller (6×10^2 kb) (18, 19). Our results tend to support the second measurement, although they do not necessarily disagree with the first one.

The C_4A_2 repeat has been found in at least three species of *Tetrahymena* (9) and probably exists widely in this group of organisms. Using the same approaches described here, we have also found this repeat in *Paramecium tetraurelia*, where it is also located near free DNA ends in the macronucleus (unpublished data). Recently the same sequence was found in another holotrich, *Glaucoma* (11). The macronuclear DNA in this ciliate is fragmented to sizes smaller than in *Tetrahymena*, and C_4A_2 repeats were found in most of the free ends by a direct sequence determination method. A similar repeated sequence (C_4A_4) was also found at the free ends of the nearly gene-sized DNA of several hypotriches (10, 20). The simple repeated sequence apparently plays an important role in the fragmentation of the ciliate chromosome.

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